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The Complete Amino Acid Sequence of the Low Molecular Weight Cytosolic Acid Phosphatase*

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This paper presents the complete amino acid sequence of the low molecular weight acid phosphatase from bovine liver. This isoenzyme of the acid phosphatase family is located in the cytosol, is not inhibited by L-(+)-tartrate and fluoride ions, but is inhibited by sulfhydryl reagents. The enzyme consists of 157 amino acid residues, has an acetylated NH_2 terminus, and has arginine as the COOH-terminal residue. All 8 half-cystine residues are in the free thiol form. The molecular weight calculated from the sequence is 17,953. The sequence was determined by characterizing the peptides purified by reverse-phase high performance liquid chromatography from tryptic, thermolytic, peptic, *Staphylococcus aureus* protease, and chymotryptic digests of the carboxymethylated protein. No sequence homologies were found with the two known acylphosphatase isoenzymes or the metalloproteins porcine uteroferrin and purple acid phosphatase from bovine spleen (both of which have acid phosphatase activity). Two half-cystines at or near the active site were identified through the reaction of the enzyme with [^{14}C] iodoacetate in the presence or in the absence of a competitive inhibitor (*i.e.* inorganic phosphate).

Ac-A E Q V T K S V L F V C L G N I C R S P I A E A V F R
K L V T D Q N I S D N W V I D S G A V S D W N V G R S P
N P R A V S C L R N H G I N T A H K A R Q V T K E D F V
T F D Y I L C M D E S N L R D L N R K S N Q V K N C R A
K I E L L G S Y D P Q K Q L I I E D P Y Y G N D A D F E T
V Y Q Q C V R C C R A F L E K V R-OH

Acid phosphatases (orthophosphoric-monoester phosphohydrolases (acid optimum), EC 3.1.3.2) are ubiquitous in nature and often occur in multiple forms differing in M_r, substrate specificity, and sensitivity to inhibitors (1–4). In addition, most of these enzymes are glycoproteins and some are also metalloproteins (such as the iron-containing acid phosphatases, porcine uteroferrin and purple acid phosphatase from spleen (5) and bone (6, 7), and the manganese-containing acid phosphatases isolated from some plants (8, 9)). The presence in mammalian tissues of low M_r isoenzymes was clearly demonstrated by Heinrikson (2) who purified the enzyme from bovine liver. Subsequently, De Araujo *et al.* (4) localized the low M_r acid phosphatase in the cytosol.

Lawrence and Van Etten (10) have recently reinvestigated the low M_r acid phosphatase from bovine liver. They reported that they had purified the enzyme to homogeneity and criticized Heinrikson's (2) data, particularly with respect to the amino acid composition and specific activity, which they found to be approximately twice as high.

The substrate specificity of the low M_r acid phosphatases is more restricted than that of the high M_r acid phosphatases in that the former efficiently hydrolyzes only *p*-nitrophenyl phosphate and riboflavin phosphate (2, 11). In 1980, we reported that the enzyme isolated from bovine liver is catalytically very active with acylphosphates such as carbamoyl phosphate and benzoyl phosphate (12). Taga and Van Etten (11) also found that the low M_r isoenzyme from human liver has a high activity on acetyl phosphate and suggested a similarity between the low M_r acid phosphatases and another class of enzymes called acylphosphatases (EC 3.6.1.7). The latter enzymes were extensively studied in our laboratory (13–16): although they have in common a subcellular localization in the cytosol and similar molecular weights, we found that the specificity of the two known isoenzymes of acylphosphatase is limited to acylphosphates and that they do not hydrolyze orthophosphoric-monoesters. In addition, the amino acid sequence was determined for several acylphosphatases from skeletal muscle of vertebrate species (17–23) and for the isoenzyme from human erythrocytes (24).

Recently, Chernoff and Lee (25) demonstrated that the major phosphotyrosyl-protein phosphatase from bovine heart is associated with a low M_r acid phosphatase. They reported that this enzyme appears to be similar to the low M_r acid phosphatases from other tissues, including the liver enzyme. In fact, all these enzymes have similar M_r, pH optima, and K_m values for *p*-nitrophenyl phosphate, and all are insensitive to inhibition by L-(+)-tartrate and fluoride ions. Furthermore, Boivin and Galand (26) purified two isoenzymes from human red cell cytosol that efficiently dephosphorylate the membrane protein band 3, previously phosphorylated on a specific tyrosine residue by a tyrosine phosphokinase present in the red cell membrane.

EXPERIMENTAL PROCEDURES AND RESULTS[1]

DISCUSSION

The complete amino acid sequence of the low molecular weight acid phosphatase (cytosol) from bovine liver is presented in Fig. 1, together with the peptides used to delineate the primary structure. The protein, consisting of 157 amino acid residues, is acetylated at the NH_2 terminus, and has Arg

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[1] Portions of this paper (including "Experimental Procedures." "Results," Figs. 2–11, and Tables I–VI) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

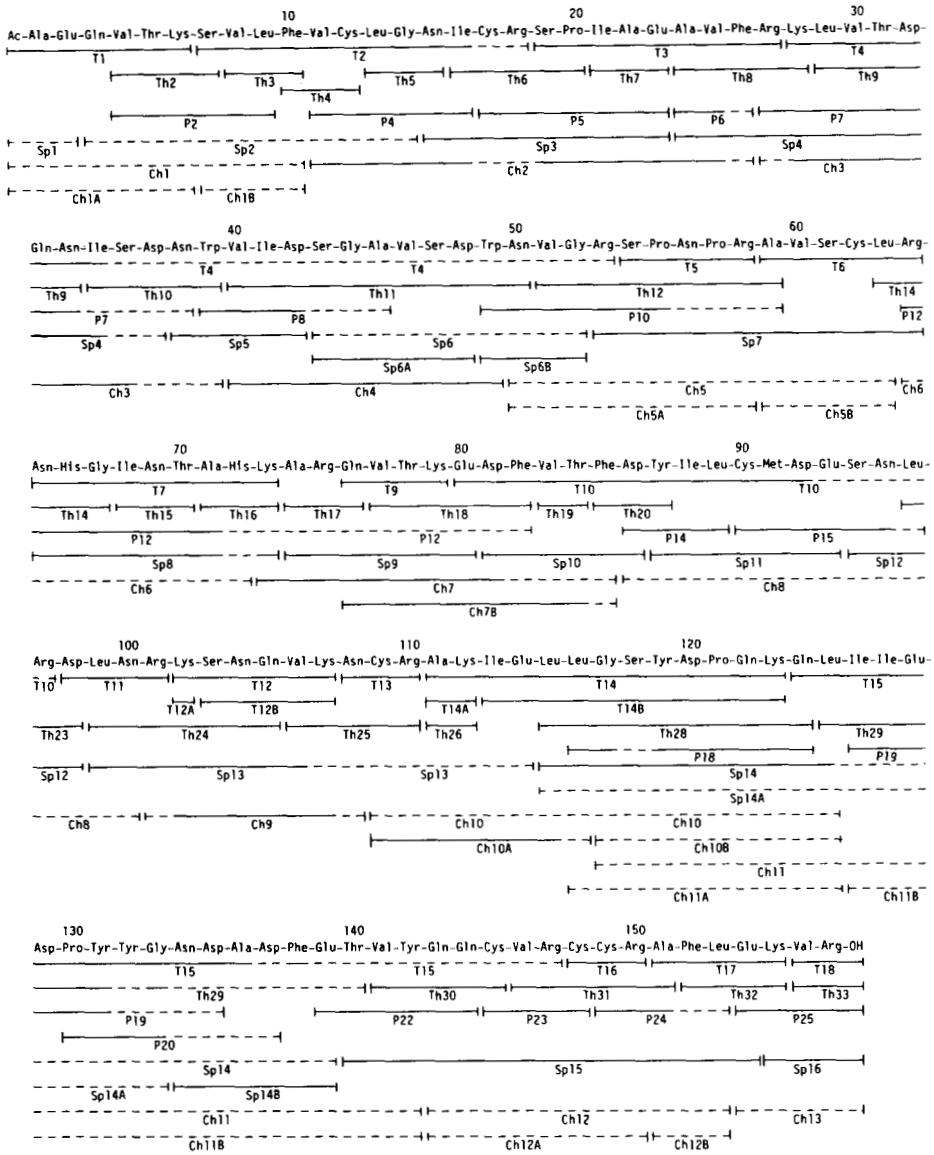

FIG. 1. **The complete amino acid sequence of the low molecular weight acid phosphatase from bovine liver.** The *solid lines* indicate the amino acid sequences determined for peptides obtained from trypsin (*T*), thermolysin (*Th*), pepsin (*P*), *S. aureus* protease (*Sp*), and chymotrypsin (*Ch*) cleavages. The notations *A* and *B* refer to the NH_2- and COOH-terminal subfragments, respectively, of a peptide which was also found unbroken. *Dashed lines* indicate sequence information which was inferred from the amino acid composition of the peptide and from data on sequence analysis of other peptides. *Ac*, acetyl.

as the COOH-terminal residue. All 8 half-cystines in the acid phosphatase are present as free sulfhydryls. The calculated minimum molecular weight is 17,953.

The protein was first reduced and carboxymethylated to stabilize the cysteine residues. The sequence was determined by analyzing the peptides obtained from five different enzymatic digestions (trypsin, thermolysin, pepsin, *S. aureus* protease, and chymotrypsin). Peptides were purified by HPLC[2] on Aquapore RP 300 with a trifluoroacetic acid/acetonitrile-based solvent system. Peaks containing more than one peptide were rechromatographed on the same column with a different solvent system and/or different elution programs.

The sequences were analyzed by the manual Edman degradation. The structure of T1, that is, the NH_2-terminal blocked peptide, was obtained by the combination of FAB mass spectrometry, enzymatic digestions, and Edman degradation, as described in the Miniprint.

The COOH-terminal Arg was determined by treatment of the Cm-protein with carboxypeptidase B. All cleavage points in the protein were overlapped by peptides obtained from one or more of the other digests.

No homology emerged when the sequence of the low M_r acid phosphatase from bovine liver was compared with that of acylphosphatase from bovine skeletal muscle (22), which indicates that these enzymes are expressed by different genes. Nor was there any homology between acid phosphatase and the isoenzyme of acylphosphatase isolated from human erythrocytes (24). The latter enzyme differs from that of human skeletal muscle in about 44% of the amino acid positions, but they clearly have originated from a common ancestral gene (24). Both of these isoenzymes show a strict specificity for acylphosphates and do not hydrolyze orthophosphoric-monoesters. Thus, although the low M_r acid phosphatase from bovine liver hydrolyzes similar acylphosphate substrates as other acylphosphatases, these share no structural similarities. Hunt *et al.* (5) have studied the sequences of two metalloglycoproteins, uteroferrin from porcine uterus and purple acid phosphatase from beef spleen, both of which exhibit acid phosphatase activity. Although their sequence data were incomplete, they demonstrated that the sequence homology between these two proteins was >90%. Comparison of these

[2] The abbreviations used are: HPLC, high performance liquid chromatography; Cm, carboxymethyl; PITC, phenylisothiocyanate; TFA, trifluoroacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; FAB, fast atom bombardment; PTH, phenylthiohydantoin; Ac, acetyl.

partial sequences (accounting for about 90% of the entire molecules) with the sequence of the low M_r acid phosphatase from bovine liver shows virtually no sequence homologies. Furthermore, the two proteins (uteroferrin consists of a single polypeptide of 35 kDa, whereas purple acid phosphatase consists of two polypeptide chains of 20 kDa and 15 kDa) have a low cysteine content: uteroferrin contains 2 cysteine residues per molecule, whereas the 20-kDa purple acid phosphatase chain contains only 1, and the 15-kDa purple acid phosphatase chain contains 2. In contrast, the low M_r acid phosphatase contains 8 cysteines per molecule (M_r = 17,953). The sequences around cysteine residues in uteroferrin and purple acid phosphatase are different from those around the 8 cysteines of the low M_r acid phosphatase. Uteroferrin and the two chains of purple acid phosphatase have free α-NH_2 groups at the NH_2 termini, whereas the low M_r acid phosphatase has an α-*N*-acetylated NH_2-terminal residue. Acetylation at the NH_2 terminus has been postulated to be characteristic of proteins synthesized on free polysomes in the cytosol (27). Thus, our results agree with the data of De Araujo *et al.* (4) on the cytosolic localization of the low M_r acid phosphatase. We found that iodoacetate causes the inactivation of the enzyme and that the competitive inhibitor P_i protects the low M_r acid phosphatase against inactivation (Fig. 10). In agreement with the data of Lawrence and Van Etten (10), our results indicate that at least 1 half-cystine residue is present at or near the active site because 85% inactivation of the enzyme occurred with the carboxymethylation of 0.9 residue of half-cystine per molecule of enzyme (Table I); furthermore, the competitive inhibitor P_i reduced the rate of inactivation by preventing the iodoacetate reaction with active site sulfhydryl group(s). Because of the difference in inactivation of the enzyme by iodoacetate in the presence and absence of P_i (Fig. 10), differential modification by [^{14}C]iodoacetate (28) was used to distinguish essential active site half-cystines from others that might be modified at the same time at other sites of the molecule. We found that Cys-12 and Cys-17, both labeled by [^{14}C]iodoacetate, are protected to the same extent by P_i, so that these two half-cystine residues are at or near the active site of the enzyme. Nevertheless, Cys-12 reacts with iodoacetate 3.5 times faster than Cys-17 (see the specific radioactivity of Cys-12- and Cys-17-containing peptides in the Miniprint). Thus, Cys-12 contributes most to enzyme's inactivation. In this paper, we present the first complete amino acid sequence for an acid phosphatase.

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Supplementary Material to:

"The Complete Amino Acid Sequence of the Low Molecular Weight Cytosolic Acid Phosphatase" by Guido Camici, Giampaolo Manao, Alessandra Modesti, Massimo Stefani and Giampietro Ramponi

EXPERIMENTAL PROCEDURES

Materials. Low M_r acid phosphatase was prepared essentially as described by Lawrence and Van Etten (10). With their technique however, we were not able to obtain a pure protein; a small quantity of a contaminating protein (about 14 KDa) always remained. Therefore we purified the enzyme to homogeneity by means of HPLC on an Aquapore RP 300 (Brownlee Labs. Inc.) column with a TFA/acetonitrile gradient system and checked its purity by SDS-PAGE or by PAGE in urea-containing gels, as described by Manao et al. (14).

Diphenylcarbamylchloride-treated trypsin, pepsinogen, carboxypeptidase Y, diisopropylfluorophosphate-treated carboxypeptidase B and α-chymotrypsin were obtained from Sigma. S.aureus V8 protease was obtained from Miles. Thermolysin was purchased from Merck (Darmstadt). Reagents and solvents (sequenal grade) for sequence determination by the Edman degradation technique were obtained from Fluka A.G. Iodo [2-^{14}C]acetic acid was from Amersham Int., with a specific radioactivity of 56 mCi/mmol.All other reagents used were of the highest purity commercially available.

Determination of free sulfhydryl groups. Total sulfhydryl group content of acid phosphatase was determined both by spectrophotometric titration with 5,5'-dithiobis(2-nitrobenzoate), Ellman's reagent (29), and by carboxymethylation of the enzyme with iodoacetate in the presence of 6 M guanidinium chloride but in the absence of reducing agents. The spectrophotometric titration was carried out by dissolving the protein in 1 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 0.01 M EDTA and 6 M guanidinium chloride; the mixture was incubated at room temperature for 20 min prior to addition of Ellman's reagent. ε_{412} = 13,600 M^{-1} cm^{-1} was used for calculation. Protein concentration was determined by amino acid analysis. The carboxymethylation of the enzyme in the absence of reducing agents was carried out as follows: the enzyme (29 nmol) was dissolved in 40 μl of 0.1 M tris-HCl buffer, pH 8.50, containing 6 M guanidinium chloride and the reaction vessel was flushed with nitrogen. After 20 min incubation at room temperature, 40 μl of 64.5 mM iodoacetic acid solution (adjusted to pH 8.70 with tris base) was added. The reaction was carried out under nitrogen atmosphere for 15 min and the carboxymethylated protein was immediately isolated from reagents by HPLC on Aquapore RP 300 (4.6 x 250 mm, 7 μm) using a TFA / acetonitrile based solvents system. Then the protein was hydrolyzed in 6 N HCl and the amino acid composition was determined.

Reduction and carboxyethylation. This process was carried out on 0.35 μmol of the low M_r acid phosphatase by the technique previously described (30) except that the carboxynethylated protein was purified from urea and reagents by gel filtration on a column (2 x 40 cm) of Sephadex G 25 superfine equilibrated with 0.2 M ammonia solution.

Amino acid analysis. Amino acid analyses were carried out on 0.5-2 nmol of peptides or protein by means of a Carlo Erba 3A29 amino acid analyzer equipped with an SP 4100 computing integrator (20). Cysteine was determined as Cm-cysteine and tryptophan was assayed by the method of Penke et al. (31). Alternatively, amino acid analysis of peptides (20-500 pmol) was performed by analyzing the phenylthiocarbamyl-derivatives of amino acids by HPLC on a Waters Pico.Tag amino acid analysis column (3.9 x 150 mm, 4 μm), using an acetonitrile gradient.

Enzymic hydrolysis. Before the various enzymatic digestions, the carboxymethylated acid phosphatase solution in a screw-cap sealed vial was immersed for 5 min in a boiling water bath and then chilled in ice.

Tryptic, peptic, S. aureus protease, and thermolytic digestions were carried out as previously described (26). Chymotryptic digestion of Cm-acid phosphatase (40 nmol) was carried out with a 2.5 % (w/w) chymotrypsin in 0.2 M ammonium bicarbonate buffer, pH 8.50, for 4 h at 37°C.

With regard to the digestion with carboxypeptidases, Cm-acid phosphatase (20 nmol) was dissolved in 0.2 M N-ethyl-morpholine-acetate buffer, pH 8.50, and mixed with carboxypeptidase B at a carboxypeptidase/substrate ratio of 0.02 units per nmol. For the digestion with carboxypeptidase Y, the peptide (6 nmol) was dissolved in 0.1 M pyridine-acetate buffer, pH 5.60, and mixed with carboxypeptidase Y at a carboxypeptidase/substrate molar ratio of 1/25. The mixtures were incubated at 30°C; aliquots were withdrawn at different time intervals, and added to 0.25 ml of 2 % sulfosalicylic acid, then centrifuged and submitted to amino acid analysis.

Fractionation of peptides. Tryptic, thermolytic, peptic, S. aureus protease and chymotryptic peptides were purified by reverse phase HPLC on an Aquapore RP 300 column (4.6 x 250 mm, 10 μm; guard column: 4.6 x 30 mm, 10 μm) with TFA/acetonitrile gradients. All peaks, except the large initial one, were rechromatographed on the same column but using 0.02 M ammonium bicarbonate/acetonitrile gradients.

COOH-terminal analysis. Carboxypeptidase B digestion was employed for the COOH-terminal analysis (see Enzymic hydrolysis).

NH_2-terminal analysis and sequence determination. Edman degradation: protein (6 nmol) or peptides (1-10 nmol) were submitted to Edman degradation carried out by the manual technique devised by Tarr (32), modified as follows: the conversion from anilinothiazolinones to PTH-derivatives was performed by incubating the dried thiazolinone extract at 80°C for 10 min with 0.2 ml of 1 N aqueous HCl, containing ethanethiol (1 % v/v); then most of the PTH-derivatives were extracted twice with 0.5 ml of ethylacetate. The organic and aqueous phases were separately dried and analyzed. PTH-derivatives present in the organic phase were analyzed by HPLC on a Beckman Ultrasfere ODS column (4.6 x 250 mm, 10 μm) according to Brown et al. (33). The analyses of PTH-His and PTH-Arg (aqueous phase) were carried out by HPLC on a small column of Aquapore RP 300 (4.6 x 250 mm, 7 μm). The elution employed the isocratic mode (90 % 0.04 M sodium acetate buffer, pH 4.40, and 10 % methanol, at a flow rate of 1 ml/min).

FAB mass spectrometry. FAB mass spectra on peptide T1 were obtained with a VG-Analytical 70-70 EQ instrument as described by Camici et al. (18).

Nomenclature of peptides. Peptides are indicated by prefixes corresponding to the type of cleavage by which they are produced: T, tryptic; Th, thermolytic; P, peptic; Sp, S. aureus protease, and Ch, chymotryptic peptides.

Chemical modification with iodoacetate. Before using, the enzyme was precipitated with 75 % ammonium sulfate, centrifuged at 10.000xg for 10 min, and the precipitate was dissolved in the buffer used for enzyme modification. The specific activity was 110 units/mg of protein (one unit of activity is defined as the amount of enzyme which catalizes the hydrolysis of 1 μmol of p-nitrophenyl phosphate per min at 37°C). Enzyme modification experiments with iodoacetic acid were performed in 0.13 M cacodylate buffer, pH 6.30, in a final volume of 2.37 ml and 25°C. Four incubation mixtures were prepared. One was 8.4 mM in iodoacetate, a second was 8.4 mM either in iodoacetate and Pi, and a third was 8.4 mM in iodoacetate and 42 mM in Pi. The control contained only enzyme and buffer. About 20 nmol of enzyme was used for each mixture. At various time intervals, 5 μl aliquots were taken and the activity was measured at pH 5.50 and 37°C with 4 mM p-nitophenylphosphate as substrate.

Isolation of ^{14}C-carboxymethylated peptides. The enzyme (40 nmol) was inactivated as described above but with ^{14}C-iodoacetate whose specific radioactivity was 0.59 nCi/nmol. After 140 min, the residual activity was about 15 % and the enzyme was purified from reagents by Sephadex G 25 chromatography (2 x 30 cm column). The protein fractions were pooled, freeze-dried, and tested for amino acid composition and radioactivity. Subsequently the protein was dissolved in 80 ul of 0.1 M tris-HCl buffer, pH 8.50 , containing 6 M guanidinium chloride and then treated with 4 ul of 2-mercaptoethanol at 40°C for 16 h. Then 80 ul of 0.645 M iodoacetic acid solution, adjusted to pH 8.70 with tris base, was added. After 15 min at room temperature the completely carboxymethylated protein was purified by HPLC on Aquapore RP 300 with TFA/acetonitrile based solvent system. Tryptic hydrolysis of the ^{14}C-carboxymethylated protein was carried out as described. The peptides were separated by HPLC as previously described, and all fractions were tested for radioactivity. Only one tryptic peptide (T2) was labelled. Because this peptide contains Cys_{12} and Cys_{17}, it was digested with thermolysin. The peptide (6 nmol) was dissolved in 40 ul of 0.1 M ammonium bicarbonate buffer, pH 8.0, containing 1 mM $CaCl_2$ and treated with 4 % thermolysin (w/w) at 37°C for 210 min. The thermolytic subfragments were separated by HPLC on Aquapore RP 300. All fractions were tested for ^{14}C-radioactivity.

RESULTS

The low M_r acid phosphatase used for the study of the primary structure was a pure protein as checked by PAGE and SDS-PAGE. The amino acid composition and the NH_2- and COOH-terminal analyses of the Cm-enzyme are presented in Table I. The amino acid composition is similar to that reported by Lawrence and Van Etten for the same enzyme (10) and is in good agreement with the amino acid composition calculated from the sequence which shows 8 half cystines. The titration with 5,5'-dithiobis(2-nitrobenzoic acid) revealed 7.6 equivalents of free sulfhydryl groups per molecule of enzyme. The carboxymethylation of the enzyme in the presence of 6 M guanidinium chloride but in the absence of reducing agents agreed closely to the results obtained with Ellman's reagent (Table I). Both these findings indicate that all eight half cystine residues occur in the form of free thiol side chains. Furthermore, Table I shows that the NH_2-terminus is not available for the PITC reagent. Arginine was found as the COOH-terminal residue by carboxypeptidase B time-course analysis. The results, expressed as mol ratios, were as follows: 10 min, 0.24 Arg; 30 min, 0.86 Arg; 2 h, 0.89 Arg; 17 h, 1.00 Arg. These results agree with the amino and carboxyl termini found in the reconstructed primary structure of the enzyme (Fig. 1).

Purification and characterization of peptides obtained by enzymic hydrolysis. Fig. 2 shows the preparative HPLC finger print of tryptic peptides. The chromatogram was developed by a gradient of 0.01 M TFA in acetonitrile as indicated in the figure legend, using an Aquapore RP 300 column. All peaks were rechromatographed on the same column but with a solvent system consisting of: a) 0.02 M ammonium bicarbonate, pH 7.0; b) acetonitrile. The gradient program for acetonitrile concentration was the same as that indicated in Fig 2. The combination of these two HPLC separations permits the purification of most of the tryptic peptides. Some hydrophilic tryptic peptides were eluted together in the large initial peak. The fractions making up this peak were pooled, dried in a vacuum concentrator (Savant), and then treated with PITC under the conditions used for the coupling step of the Edman degradation. The mixture of phenylthiocarbamoyl-tryptic peptides was separated by HPLC on Aquapore RP 300 using a solvent system composed of 0.02 M ammonium bicarbonate, pH 7.0, and acetonitrile (Fig. 3). The fractions corresponding to the peaks were dried in separate mini-reactors and then exposed to an acidic environment for the cleavage step of Edman degradation, and all subsequent operations needed to obtain the PTH-derivatives were carried out. These derivatives were analyzed by HPLC and the Edman degradation was continued on the residual peptides to obtain sequence information. Table II reports the amino acid compositions and yields of all purified tryptic peptides. The sequence information for all tryptic peptides is reported in Fig. 1. Fig. 4 shows the preparative HPLC finger print for the thermolytic peptides. The thermolytic peaks were also rechromatographed under the same conditions described for the tryptic peptides. In addition the big initial peak was treated as described for the tryptic peptides (Fig. 5 reports the HPLC separation of the phenylthiocarbamoyl-thermolytic peptides) to obtain sequence information on its hydrophilic peptides. The amino acid compositions and yields of the purified thermolytic peptides are shown in Table III, while the sequence information is given in Fig. 1. Figs. 6-8 report the preparative HPLC finger prints obtained from the peptic, S. aureus protease and chymotryptic hydrolyses, respectively, of the Cm-low M_r acid phosphatase. The legends of these figures describe the experimental conditions . All peaks were rechromatographed under the conditions described for the tryptic peptides. With respect to these hydrolysates we did not consider it necessary to characterize the hydrophilic peptides in the

large initial peaks. Tables IV-VI report the amino acid compositions of these last three peptide series, whose sequence information is presented in Fig 1. Two peptides (Sp2 and Sp15) partially precipitated during the digestion and were separated prior to fractionation of the mixture on the Aquapore RP 300 HPLC column. The precipitate was dissolved in 6 M guanidinium chloride; thus Sp2 and Sp15 were purified by HPLC on Aquapore RP 300 using the TFA-acetonitrile solvent system and the same gradient elution used for other Sp peptides. Two anomalous cleavages were observed for P20 (Asp_{129}-Pro_{130} bond) and P19 (Asp_{135}-Ala_{136} bond). The cleavage at the Asp-Pro bond was probably due to the low pH value and the relatively long incubation time at 37°C during peptic digestion. In fact Hermodson (34) reported that aspartyl-prolyl bonds are (in some cases) susceptible to cleavage in acidic solutions. As for the partial cleavage at the Asp-Ala bond, it is most likely derived from non-specific digestion, as indicated by its low yield. Kasper (35) has reported that the pepsin susceptible bonds in proteins are those formed by the carboxyl group of all L-amino acids except proline.

On the basis of the sequence information from the various peptide series and the amino acid composition of all purified peptides we were able to reconstruct the complete amino acid sequence (Fig. 1).

Structure of T1. This peptide represents the NH_2-blocked (Edman-negative) fragment obtained by tryptic hydrolysis of the Cm-enzyme. Its structure was obtained by the combination of the following techniques: i) FAB mass spectrometry of the entire peptide; the FAB mass spectrum in Fig. 9 shows a protonated molecular ion at m/z 717, a value which corresponds to a M_r of 716; ii) digestion of T1 with *S. aureus* protease which generated the NH_2-acylated T1Sp1 and the T1Sp2 fragments. These peptides were separated by a small column (3 x 30 mm) of AG50W-X4 (H^+-form) resin (Bio-Rad). The fragment T1Sp1 was not retained by this column, whereas the fragment T1Sp2 was eluted by 3 M ammonia solution. The amino acid compositions of these peptides were: T1Sp1: Ala(0.9), Glu(1.0); T1Sp2: Thr(0.9), Glu(1.0), Val(0.9), Lys(1.0). The digestion of the NH_2-acylated T1Sp1 with carboxypeptidase Y released only Glu, which is assigned to position 2 (this result was expected in view of the specificity of staphylococcal protease). The absence of Ala in the carboxypeptidase Y hydrolysate, which should have appeared in an amount equimolecular to that of Glu, indicates that Ala is the NH_2-terminal acylated residue.

T1Sp2 was sequenced by Edman degradation and gave the following results: Gln-Val-Thr-Lys.

The amino acid composition of T1 (Table II) and the above data indicate the presence of an acetyl group blocking the NH_2-terminus.

In conclusion the structure of T1 was: Ac-Ala-Glu-Gln-Val-Thr-Lys.

Glutamine found at position 3 agrees with the data on the M_r of T1, calculated from the FAB mass spectrum.

Active site modification. Fig. 10 shows the inactivation experiments of the low M_r acid phosphatase by iodoacetate. The kinetic is pseudo-first order both in the presence and in the absence of Pi, a competitive inhibitor of the enzyme. Inorganic phosphate protects the enzyme against inactivation and these results agree with those reported by Lawrence and Van Etten (10) on the same enzyme, from which we conclude that at least one half cystine residue occurs at the active site of the enzyme. When 85 % inactivation had occurred (after about 140 min.) further modification was prevented by quickly separating the enzyme from reagents and salts with gel filtration on Sephadex G 25. Then amino acid analysis was carried out on the modified protein. The results reported in Table I indicate that 85 % of enzyme activity loss was related to the carboxymethylation of 0.9 residues of half cystine per molecule of enzyme, while no loss of other amino acid residue was observed. This seems to indicate that one half cystine is involved in the active site of the enzyme. We therefore proceeded to localize the half cystine residue(s) that could be labeled with ^{14}C-iodoacetate at about 85 % inactivation of the enzyme. The labelled enzyme was then completely carboxymethylated by unlabelled iodoacetate and the Cm-protein was purified by HPLC as described in the "Experimental Procedure" Section. Then the Cm-protein was digested with trypsin. Only T2 incorporated ^{14}C-radioactivity. This peptide contains both Cys_{12} and Cys_{17}. T2 was then digested with thermolysin and the subfragments were separated by HPLC. Figures 11A,B show the chromatogram and the radioactivity, respectively. Only three peaks were ^{14}C-labelled; these were hydrolyzed and their amino acid composition and sequence revealed that T2Th1 (specific radioactivity = 0.36 nCi/nmol) corresponds to Val-Cys_{12}, T2Th2 (specific radioactivity = 0.34 nCi/nmol) corresponds to Phe-Val-Cys_{12} and T2Th3 (specific radioactivity = 0.10 nCi/nmol) corresponds to Leu-Gly-Asn-[Ile-Cys_{17}]-Arg. The mean specific radioactivity calculated for Cys_{12} was 0.35 nCi/nmol, whereas Cys_{17} had a specific radioactivity of 0.10 nCi/nmol, 3.5 times lower than that of Cys_{12}. These data demonstrate that the modification of Cys_{12} was responsible for the loss of most of the enzymatic activity, although Cys_{17} also reacted but to a lower extent. Cys_{12} and Cys_{17} are close enough to each other so that it appears possible that both were present at or near the active site of the enzyme. To clarify this point we labelled the active site cysteine(s) with ^{14}C-iodoacetate but in the presence of 40 mM Pi. This Pi concentration protects the enzyme almost completely against inactivation by iodoacetate (Fig. 10). The enzyme was processed in the way described above for the modification of the enzyme in the absence of Pi. The thermolytic peptides from T2 were checked for radioactivity and the results, reported in Fig. 11C, clearly demonstrate an efficient protective action by Pi which competes with iodoacetate at the active site of the enzyme. This protection was comparable for all three peaks. Since the radioactivity bound to peptides T2Th1, T2Th2, and T2Th3 was 14 %, 14 %, and 15 % respectively, as referred to data obtained in the absence of Pi.

Table I. Amino Acid Composition and Terminal Residues of Bovine Liver Low Molecular Weight Cm-Acid Phosphatase .

Amino acid[a]	Reduced and carboxymethylated	Carboxymethylated without reduction	Sequence values	Modified[e]	Modified, Pi[f]
Cm-Cysteine	7.9	7.6	(8)	0.9	0.2
Aspartic acid	21.0	23.2	(24)	23.3	22.7
Threonine	5.6	6.2	(6)	5.9	5.7
Serine	9.0	9.8	(10)	9.7	10.3
Glutamic acid	15.8	16.7	(16)	16.8	16.9
Proline	4.9	4.8	(5)	n.d.	n.d.
Glycine	5.5	6.2	(6)	6.4	6.5
Alanine	9.2	9.5	(10)	9.1	9.4
Valine	13.1	14.2	(15)	15.2	14.5
Methionine	0.8	1.0	(1)	1.1	1.1
Isoleucine	7.8	8.5	(9)	8.5	8.2
Leucine	10.7	11.3	(11)	11.0	11.0
Tyrosine	5.1	4.9	(5)	5.1	5.0
Phenylalanine	6.4	6.1	(6)	6.2	5.8
Lysine	9.2	9.1	(9)	9.0	8.8
Histidine	2.0	2.0	(2)	2.1	2.2
Arginine	12.7	12.2	(12)	11.7	11.6
Tryptophan[b]	1.8	n.d.	(2)	n.d.	n.d.

NH_2-Terminus: Edman[c] : none
COOH-Terminus: Carboxypeptidase B[d] : Arg

[a] The results are expressed as residues per molecole of enzyme. Values for Ser and Thr were determined by hydrolyses at 110°C for 22 and 70 h in duplicate and extrapolation to zero time. [b] Determined according to (29). [c] The NH_2-terminal analysis was carried out on 5 nmol of Cm-acid phosphatase. [d] The carboxypeptidase B digestion was performed on 30 nmol of Cm-acid phosphatase. [e] 85 % inactivated by iodoacetate in 140 min. [f] In the presence of 42 mM Pi, 12 % inactivated by iodoacetate in 140 min. n.d., not determined.

Table II. Amino Acid Composition of Tryptic Peptides from Bovine Liver Low Molecular Weight Cm-Acid Phosphatase[a].

	Cm-Cys	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp	Yield %	Sequence position
T1			1.1		1.0			0.8	0.9						1.0				33	1-6
T2	1.0	1.2		0.8	0.3		1.0		2.2		1.0	1.8	0.2	1.0			1.0		35	7-18
T3				0.8	1.0	1.0		1.8	1.0		1.1			1.0			1.0		70	19-27
T4		6.5	1.1	3.1	1.3		2.4	1.3	4.0		1.8	1.2			1.1		1.2	1.8	26	28-53
T5		1.1		0.7		1.0											1.0		57	54-58
T6	1.2			0.8				0.8	1.0				0.9				1.0		43	59-64
T7		1.7	1.3				1.0	0.9			1.1				1.0	1.0			56	65-73
T10	1.1	3.8	1.1	1.0	2.0				1.1	0.9	0.8	2.0	1.0	2.0			1.0		39	80-97
T11		1.8										1.0					1.1		55	98-101
T14		0.9		0.8	2.0	1.0	1.0	1.0			0.8	2.0	0.9		2.0				12	111-123
T14B		0.8		0.8	2.0	1.0	1.1				0.9	2.0	1.0		1.1				44	113-123
T15	1.1	4.2	1.3		5.3	1.1	1.3	1.0	2.0		1.6	1.1	3.1	1.0			1.0		11	124-147
T17					1.1			0.8				1.0		1.1	1.2				63	151-155

[a] The digestion was carried out on 50 nmol of Cm-acid phosphatase. The values are expressed as molar ratios. Values for Ser and Thr were corrected for 15 % and 5 % destruction, respectively. Values of contaminating amino acids at a level of less than 15 % are not reported. The hydrophilic peptides T9, T12, T12A, T12B, T13, T14A, T16 and T18 (see Fig. 2) were derivatized with PITC and then separated as phenylthiocarbamoyl-derivatives (see Fig. 3), and successively sequenced without determining their amino acid composition. Yield was calculated as moles obtained from moles of protein digested.

Table III. Amino acid composition of thermolytic peptides from bovine liver low molecular weight Cm-acid phosphatase[a].

	Cm-Cys	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp	Yield %	Sequence position
Th3									1.1			1.0		1.0					35	8-10
Th4	1.2								1.0					1.0					22	10-12
Th5		0.9					1.1					1.0							73	13-15
Th6	1.2			1.0		1.0					0.9						1.0		73	16-20
Th8								0.9	0.9					0.9	1.1		1.0		41	24-28
Th9		2.1	1.0		1.0				1.0			1.0							96	29-34
Th10		2.0		1.1							0.9							0.9	50	35-39
Th11		2.7		1.9			1.0	1.0	1.8		0.8							1.0	38	40-50
Th12		1.9		0.9		2.0	1.0	0.9	1.2								2.0		60	51-59
Th14		0.9					1.0					0.9				1.0	1.0		41	63-67
Th15		1.1	1.0		1.1				1.0					0.9	1.1				72	77-82
Th20		1.9											0.9	1.0					74	85-87
Th23		0.9										1.0					1.1		53	96-98
Th24		2.0		1.0	1.0							1.0			1.1		1.0		14	99-105
Th25	1.2	1.0							0.9						1.2		1.1		34	108-110
Th26		1.1		1.0	2.1	1.0	1.0					2.0	0.9		1.0				53	115-124
Th29		4.1	1.0		2.1	1.2	1.1	1.0			1.8	1.0	1.9	1.0					32	125-140
Th30	1.1				2.1				1.0				0.9						84	141-145
Th31	2.1							1.0	1.0								2.1		32	146-151
Th32					1.0							1.0		1.0	1.0				41	152-155

[a]The digestion was performed on 50 nmol of Cm-acid phosphatase. The hydrophilic peptides Th2, Th16, Th17, Th18, Th28 and Th33 (see Fig. 4) were derivatized with PITC and then separated as phenylthiocarbamoyl-derivatives (see Fig. 5), and successively sequenced without determining their amino acid composition. Other details are the same as in Table II.

Table IV. Amino Acid Composition of Peptic Peptides from Bovine Liver Low Molecular Weight Cm-Acid Phosphatase[a].

	Cm-Cys	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp	Yield %	Sequence position
P2			0.9	1.0					1.0			1.0			1.0				62	4-9
P4	0.9	1.0					1.0		1.0		1.0	1.0							16	11-16
P5	1.0		0.3	1.0	1.0	0.9		0.8	0.2		0.8						0.9		38	17-23
P6								1.0	1.0					1.0					33	24-28
P7		3.9	0.9	1.1	1.0				1.2		0.9	1.0			1.0		1.1		15	27-38
P8		1.0		1.0			1.1	1.0	0.9		1.0							0.9	41	39-45
P10		2.1		1.0		1.9	1.3	1.1	1.0								1.8	1.0	19	49-59
P12		3.2	1.7		2.1		1.0	1.9	1.1		1.0			0.9	2.1	1.8	2.0		14	64-82
P14		1.0									1.0	1.1	1.0						22	86-89
P15	0.8	2.0		1.2	1.1					0.7		1.2							37	90-96
P16		1.3		0.9	2.1	1.1	1.0					1.0	1.0		1.0				37	116-124
P19		3.0			1.0	1.1	1.0				1.6		1.9						12	126-135
P20		2.9				0.9	1.0	0.8					1.7						24	130-137
P22			1.0		3.0				1.1				0.8						60	139-144
P23	1.9								1.0								1.0		47	145-148
P24	1.1							1.0				1.0		1.0			1.0		39	149-153
P25					1.1				0.9						1.0		1.1		70	154-157

[a]The digestion was carried out on 40 nmol of Cm-acid phosphatase. Other details are the same as in Table II.

Table V. Amino acid composition of *S. aureus* V8 protease peptides from bovine liver low molecular weight Cm-acid phosphatase[a].

	Cm-Cys	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp	Yield %	Sequence position
Sp1					1.0			1.0											95	1-2
Sp2[b]	1.2	0.3	1.1	1.2	1.3		1.2		2.6			2.0		1.0	0.9				14	3-14
Sp3	1.0	0.8		0.8	1.0	0.7		1.2			2.2						0.7		21	15-23
Sp4		3.2	0.9	1.0	1.0			0.9	2.0		0.9	1.0		1.0	1.0		1.0		33	24-37
Sp5		2.1							1.0		0.9							1.0	33	38-42
Sp6		1.7		2.0			2.0	1.0	2.1									0.9	24	43-52
Sp6A		1.3		1.9	0.2		1.0	1.0	0.9	0.2									26	43-48
Sp6B		1.0					1.0		1.0									0.9	14	49-52
Sp7	0.8	1.3		2.0		2.1		0.9	1.1			1.0					3.3		11	53-64
Sp8		2.3	1.2				1.0	0.9			1.0				1.2	2.0			5	65-73
Sp9			1.0		2.0			1.0	1.2						1.0		1.2		21	74-80
Sp10		2.0	1.0						1.0					1.9					69	81-86
Sp11	1.1	1.0			1.0					1.0	1.0	1.0	0.9						32	87-93
Sp12		2.0		1.0								1.0					1.0		54	94-98
Sp13	1.2	3.3		0.7	2.2			1.0	1.1		1.0	1.1			3.0		1.5		46	99-114
Sp14		5.0		1.0	4.0	1.9	2.0	1.2			2.3	3.2	2.7	0.9	0.9				20	115-139
Sp14A		2.1		0.9	2.8	2.0	1.9				1.7	3.0	2.9		1.0				13	115-133
Sp14B		2.9			1.2			1.0						1.1					14	134-139
Sp15[b]	3.4		1.2		3.0			1.0	1.9			0.9	0.8	0.7			2.1		22	140-154
Sp16								1.0							1.0		1.1		75	155-157

[a]The digestion was performed on 60 nmol of Cm-acid phosphatase. [b]These peptides partially precipitated during digestion. Other details are the same as in Table II.

Table VI. Amino acid composition of chymotryptic peptides from bovine liver low molecular weight Cm-acid phosphatase*.

	Cm-Cys	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp	Yield %	Sequence position
Ch1			0.9	1.0	2.1			1.0	2.0			1.0		1.1	1.0				23	1-10
Ch1A			1.0		2.1			1.0	1.0						1.1				27	1-6
Ch1B		0.2		1.1					1.0			1.0		1.0					27	7-10
Ch2	1.8	1.0		1.0	1.1	0.9	1.1	1.8	2.0		2.0	1.1		1.1		1.0			48	11-26
Ch3		3.7	0.9	1.0	1.0				1.0		1.0	1.0			1.0		1.0	1.0	34	27-39
Ch4		1.9		2.0				1.2	1.1	1.9		0.8						0.9	58	40-49
Ch5	1.0	2.0		2.0		2.0	1.0	0.9	1.8			1.0					2.0		19	50-63
Ch5A		2.0		1.0		1.8	1.0		1.0								2.0		23	50-58
Ch5B	1.1			1.1				1.0	1.0			1.1							16	59-63
Ch6		2.0	1.0				1.1	1.0			1.0					2.1	1.0		14	64-72
Ch7		1.1	1.8		1.9			1.0	1.9					2.0	2.2		1.1		6	73-85
Ch7B		1.1	1.9		2.0				1.8					1.9	1.1				42	76-85
Ch8	1.0	4.7		1.0	1.0					1.0	0.9	2.9	0.9				1.0		14	86-100
Ch9		2.0		1.0	1.0				1.1						2.0		1.0		5	101-108
Ch10	0.7	1.2		1.2	3.0	0.9	1.1	1.0			1.1	3.1	0.9		2.2		1.0		4	109-125
Ch10A	1.0				1.0			0.9			1.0	2.0			1.0		1.0		13	109-116
Ch10B		1.0		1.1	2.0	1.0	1.0					1.0	1.0		1.0				20	117-125
Ch11		4.9	0.9	1.2	3.9	1.9	2.2	1.0	1.2		1.6	1.0	3.7	1.3	1.0				7	117-142
Ch11A		1.0		1.1	1.9	1.0	1.1					2.0	1.0		1.0				12	116-125
Ch11B		3.9	0.8	0.5	1.9	0.9	1.1	1.0	1.0		1.7		2.7	1.1					37	126-142
Ch12	3.3				2.0			1.0	0.9			1.0		1.0			2.0		18	143-153
Ch12A	3.2				2.0				1.1								2.0		8	143-150
Ch12B								0.9				1.0		1.0					13	151-153
Ch13					1.0				1.1						1.0		0.9		56	154-157

*The digestion was carried out on 40 nmol of Cm-acid phosphatase. Other details are the same as in Table II.

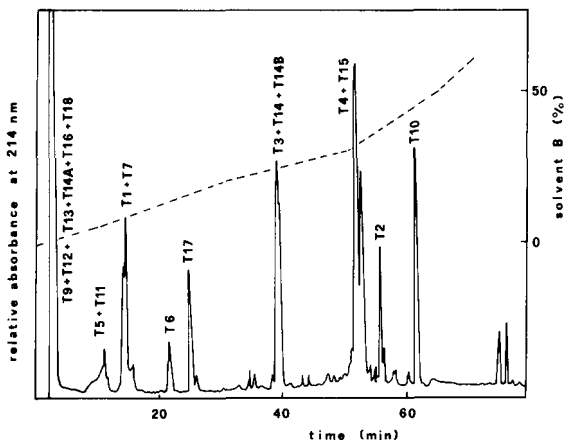

Fig. 2. **The HPLC separation of tryptic peptides from 50 nmol of the Cm-acid phosphatase.** Column: Aquapore RP 300, 10 µm, 4.6 x 250 mm; guard column: Aquapore RP 300 , 10 µm, 4.6 x 30 mm. Solvent A: 10 mM TFA in water: Solvent B: 10 mM TFA in acetonitrile. Flow rate, 1.5 ml/min; (—), absorbance; (- -), elution gradient. The overlapped peptides were rechromatographed at different conditions. The peptides contained in the big peak near the origin was first derivatized with PITC and then the phenyl-thiocarbamoyl-derivatives separated by HPLC (see Fig. 3).

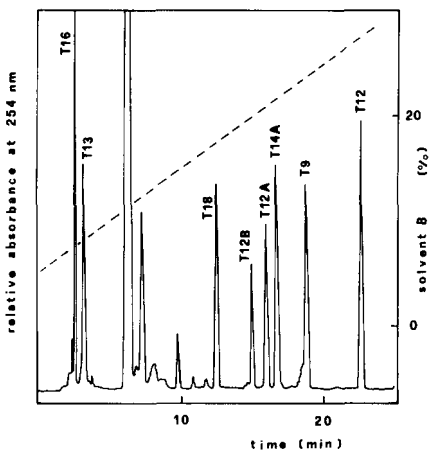

Fig. 3. **The HPLC separation of the phenylthiocarbamoyl-derivatives of the hydrophylic tryptic peptides .** Details are the same as in Fig. 2,except: solvent A: 0.02 M ammonium bicarbonate, pH 7.0;solvent B: acetonitrile.

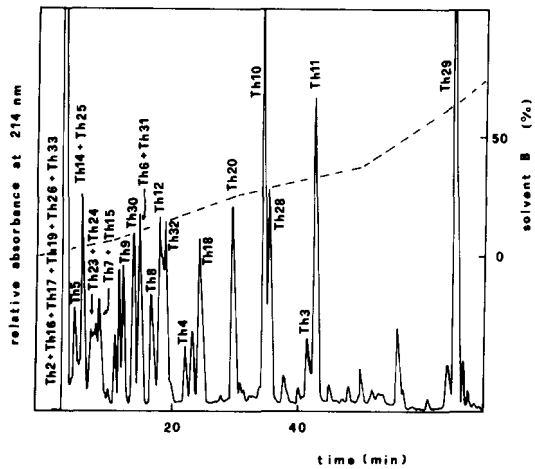

Fig. 4. **The HPLC separation of thermolytic peptides from 50 nmol of the Cm-acid phosphatase.** Details are the same as in Fig. 2.
The overlapped peptides were rechromatographed at different conditions. The peptides in the big peak near the origin were first derivatized with PITC and then separated by HPLC (Fig.5).

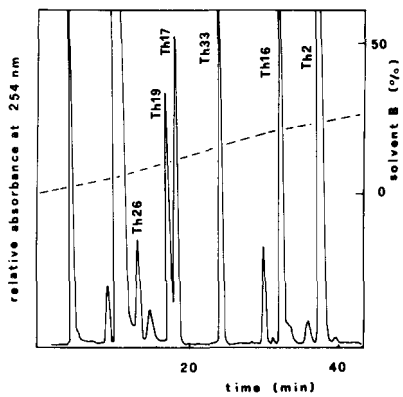

Fig. 5. **The HPLC separation of the phenylthiocarbamoyl-derivatives of the hydrophylic thermolytic peptides.** Details are the same as in Fig. 3.

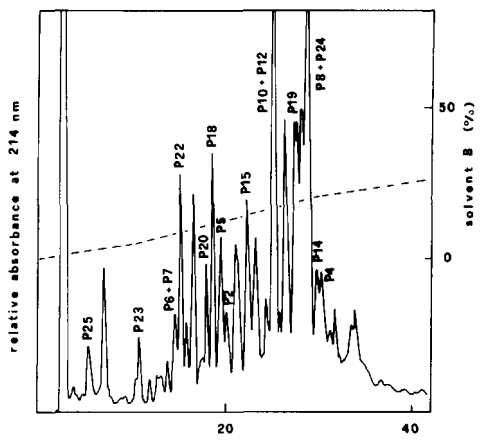

Fig. 6. **The HPLC separation of peptic peptides from 40 nmol of the Cm-acid phosphatase.** Details are the same as in Fig. 2. The overlapped peptides were rechromatographed at different conditions.

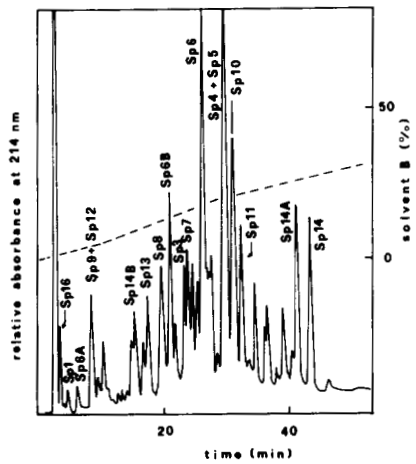

Fig. 7. **The HPLC separation of S. aureus protease peptides from 60 nmol of the Cm-acid phosphatase.** Details are the same as in Fig 2. The overlapped peptides were rechromatographed at different conditions.

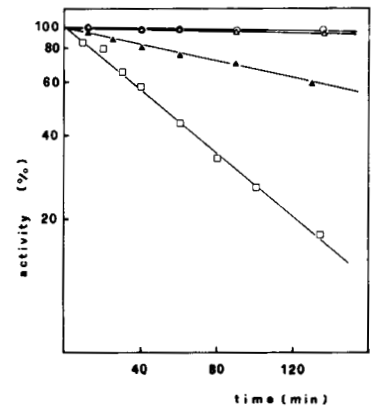

Fig. 10. **Inactivation of the low M_r acid phosphatase by iodoacetate in the presence and in the absence of Pi.** Incubations were performed in 0.13 M cacodylate buffer, pH 6.3, containing 1 mM EDTA at 25°C. ○--○ , control; □--□ , 8.4 mM iodoacetate; ▲--▲ , 8.4 mM iodoacetate and 8.4 mM Pi; Δ--Δ , 8.4 mM iodoacetate and 42 mM Pi.

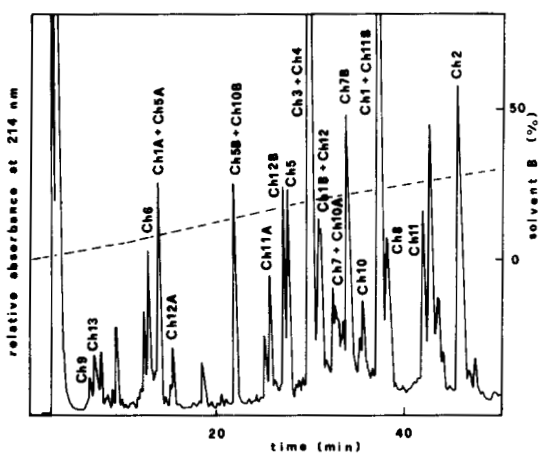

Fig. 8. **The HPLC separation of chymotryptic peptides from 40 nmol of the Cm-acid phosphatase.** Details are the same as in Fig. 2. The overlapped peptides were rechromatographed at different conditions.

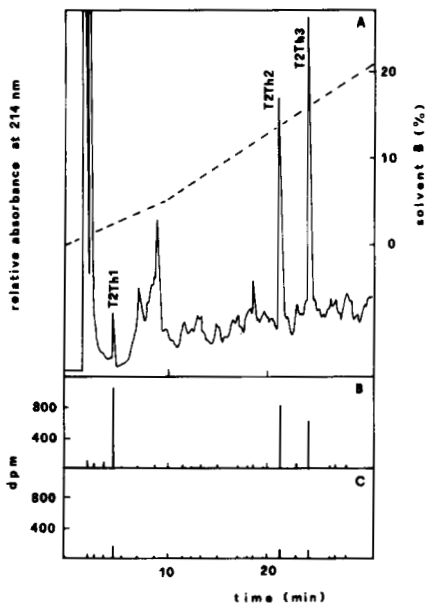

Fig. 11. **HPLC separation of the thermolytic subfragments of the peptide T2.** A, details are the same as in Fig. 7; B, ^{14}C-radioactivity measured on the chromatografic fractions obtained from the enzyme modified at the active site by ^{14}C-iodoacetate without Pi; C, ^{14}C-radioactivity measured on the chromatographic fractions obtained from the enzyme incubated with ^{14}C-iodoacetate but in the presence of 43 mM Pi.

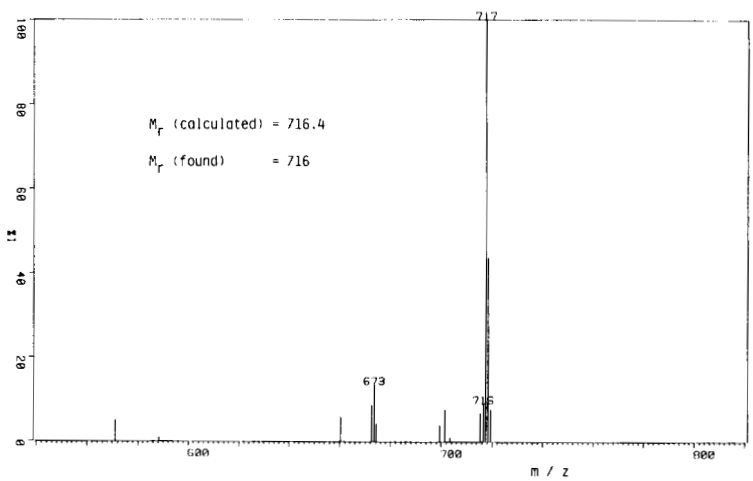

Fig. 9. **Positive FAB mass spectrum of T1.**